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Integron Content of Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* Strains over 12 Years in a Single Hospital in Madrid, Spain

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The contribution of integrons to the dissemination of extended-spectrum β-lactamases (ESBL) was analyzed on all ESBL-producing *Escherichia coli* isolates from 1988 to 2000 at Ramón y Cajal Hospital. We studied 133 *E. coli* pulsed-field gel electrophoresis types: (i) 52 ESBL-producing clinical strains (C-ESBL) (16 TEM, 9 SHV, 21 CTX-M-9, 1 CTX-M-14, and 5 CTX-M-10); (ii) 43 non-ESBL blood clinical strains (C-nESBL); and (iii) 38 non-ESBL fecal isolates from healthy volunteers (V-nESBL). Class 1 integrons were more common among C-ESBL (67%) than among C-nESBL (40%) or V-nESBL (26%) ($P < 0.001$) due to the high number of strains with $bla_{CTX\text{-}M\text{-}9}$, which is linked to an In6-like class 1 integron. Without this bias, class 1 integron occurrence would be similar in C-ESBL and C-nESBL groups (47% versus 40%). Occurrence of class 2 integrons was similar among clinical and community isolates (13 to 18%). No isolates contained class 3 integrons. The relatively low rate of class 1 integrons within transferable elements carrying bla_{TEM} (23%) or bla_{SHV} (33%) and the absence of class 2 integrons in all ESBL transconjugants mirror the assembly of translocative pieces containing bla_{TEM} or bla_{SHV} on local available transferable elements lacking integrons. The low diversity of class 1 integrons (seven types recovered in all groups) might indicate a wide dissemination of specific genetic elements in which they are located. In our environment, the spread of genetic elements encoding ESBL has no major impact on the dispersion of integrons, nor do integrons have a major impact on the spread of ESBL, except when bla_{ESBL} genes are within an integron platform such as $bla_{CTX\text{-}M\text{-}9}$.

The most obvious risk factor for the dissemination of genes encoding extended-spectrum β-lactamases (ESBL) is directional selection of resistant strains following the use of β-lactam antibiotics, particularly expanded-spectrum cephalosporins (5). Nevertheless, the investigation of other factors might be critical for predicting the potential spread and evolution of ESBL-producing strains.

Genes encoding ESBL are usually located on conjugative plasmids (such as bla_{TEM} or bla_{SHV}), although many of the most recently described ESBL genes are frequently found within integron-like structures (such as $bla_{CTX\text{-}M}$, bla_{GES}, or $bla_{VEB\text{-}1}$) (4–6, 17). On the other hand, ESBL-producing isolates are usually resistant to other antibiotics such as aminoglycosides, tetracyclines, chloramphenicol, trimethoprim, sulfonamides, or quinolones, often due to the presence of different resistance genes on transferable elements such as plasmids, transposons, or integrons and/or genetic structures generated by combinatorial evolution of different interactive pieces (2, 17, 27, 29, 39–41). The fact that ESBL genes could be acquired by strains harboring particular integrons may enlarge the possibilities of selection of these isolates by a variety of different antimicrobials. Moreover, ESBL genes can be located on integrons, which may facilitate the spread of such genetic elements (4, 6).

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Integrons are natural highly efficient recombination and expression systems able to capture genes as part of genetic elements known as gene cassettes (32). Five integron classes related to antibiotic resistance have been described based on the homology of their integrase genes (1, 3, 16, 32; Gene Bank accession no. AJ277063). Class 1 integrons are the most commonly found in nosocomial and community environments, followed by class 2 integrons, other integron classes being scarcely reported to date (12, 31, 32, 42). An increase in the rate of clinical isolates containing antibiotic resistance integrons (ARI) has been observed in several institutions during the last years, with changes in the gene cassette content of class 1 integron over time (34, 43). The widespread presence of *Enterobacteriaceae* containing ARI observed among the community-based population indicates the existence of a substantial reservoir potentially feeding multidrug resistance in the nosocomial setting (19, 23). ESBL located on integron-like structures are also being increasingly reported worldwide (4, 6). Although independent association between integrons and particular antimicrobial agents (including ampicillin and/or piperacillin and cefuroxime) has been suggested (20), the association of these genetic elements with antibiotic resistance in ESBL-producing isolates has not been previously explored.

In this study we investigated the occurrence, distribution, and cassette content of integrons among all different ESBL-producing *Escherichia coli* clones isolated from patients at the Ramón y Cajal University Hospital in Madrid (Spain) during 12 years (1988 to 2000). To evaluate the contribution of class 1, class 2, and class 3 integrons to the dissemination of the

different ESBL types (TEM, SHV, and CTX-M) and the antibiotic resistance genes frequently associated with ESBL-producing organisms, the presence of these integrons was also investigated among non-ESBL-producing *E. coli* isolates from patients attending the same hospital and from healthy volunteers.

MATERIALS AND METHODS

Bacterial strains and epidemiological background. The 133 *E. coli* isolates included in this study were divided into three groups on the basis of the source and ESBL production. Only one isolate per person was studied, and all pulsed-field gel electrophoresis-related isolates were excluded. ESBL-producing *E. coli* clinical isolates (C-ESBL, $n = 52$) were obtained from clinical specimens of patients located in different wards of our institution (Hospital Ramón y Cajal, Madrid, Spain) between 1988 and 2000 (40.4% in surgical wards, 21.1% in intensive care units, 15.4% in medical wards, and 23.1% outpatients). These isolates were recovered from urine ($n = 25$), rectal swabs ($n = 7$), respiratory samples ($n = 6$), blood ($n = 5$), wounds ($n = 5$), and other samples ($n = 4$). Strains corresponding to a control group of clinical non-ESBL-producing *E. coli* isolates (C-nESBL, $n = 43$) were obtained from blood cultures of patients at our hospital during the same period of time in surgical wards (39.5%), medical wards (18.6%), intensive care units (16.3%), and the emergency room (25.6%). Community-type non-ESBL-producing *E. coli* strains were isolated from feces of healthy volunteers living in Madrid during 2001 (V-nESBL, $n = 38$) without exposure to antibiotics or hospital environment in the 3 months previous to the sample recovery. For samples from this group, 0.5 g of feces was suspended in 0.5 ml of normal saline and 200 μl was inoculated into MacConkey agar. Only one colony per colonial morphology and resistance phenotype from each person was selected for further analysis. Preliminary susceptibility testing was performed by using the automated PASCO (Difco, Detroit, MI) or WIDER (Fco. Soria Melguizo, Madrid, Spain) system. Bacterial identification was performed using these commercial systems and/or standard biochemical tests.

Clonal and phylogenetic analysis of *E. coli* isolates. Chromosomal DNA was prepared as previously described using XbaI as a restriction enzyme (Amersham Life Sciences, Uppsala, Sweden) (18). DNA fragments were separated by electrophoresis in 1.2% agarose gels (pulsed-field agarose certified; Bio-Rad, Hemel Hempstead, United Kingdom) and 0.5× Tris-borate-EDTA buffer by using a contour-clamped homogeneous electric field (CHEF-DRIII system; Bio-Rad) and the following electrophoresis conditions: 12°C at 6 V/cm for 27 h with pulse times ranging from 10 to 40s. Clonal relationships were established following criteria by Tenover et al. (38).

A multiplex PCR assay described by Clermont et al. (8) was performed to assign phylogenetic groups among *E. coli* isolates. In this analysis, three pairs of primers were used in order to amplify the *chuA* and *yjaA* genes and an anonymous DNA fragment, which have been found to be specific phylogenetic group markers (8, 15) (Table 1).

Antimicrobial susceptibility and ESBL identification. Susceptibility to 13 non-β-lactam antibiotics was determined by the standard disk diffusion method following NCCLS guidelines (24). Disks were purchased from OXOID (Basingstoke, England). All intermediate-susceptible strains were considered nonsusceptible strains. The presence of an ESBL phenotype was presumptively determined by the standard double-disk synergy test. Characterization of ESBL was performed by isoelectric focusing, amplification of *bla* genes by PCR using primers and conditions showed in Table 1, and further sequencing of PCR products.

Conjugation of ESBL-producing isolates was performed by broth and/or filter mating standard methods using *E. coli* strain BM21 (nalidixic acid resistant, lactose fermentation positive, and plasmid free) or *E. coli* strain BM21R (a rifampin-resistant mutant of *E. coli* BM21) as the recipient (9).

Detection and characterization of class 1, 2, and 3 integrons. Detection of class 1 and class 2 integrons was performed by PCR using genomic DNA from wild-type strains and the corresponding transconjugants as the template. The primers used for detection and characterization of integrons are shown in Table 1. For class 1 integrons, two primer sets were used: IntI1-F/IntI1-R for amplifying the *intI1* gene (23) and 5′CS/3′CS for amplifying the integron variable region-containing gene cassettes (21). For class 2 integrons, the primers used were IntI2-F/IntI2-R for amplifying the *intI2* gene (23) and attI2-F/orfX-R (this study; 42) to characterize the gene cassette arrays in class 2 integrons. The primers attI2-F and orfX-R bind, respectively, to *attI2* and to *orfX*, which is situated within Tn*7* in a position just downstream of the cassette region. PCR products were separated by electrophoresis on 0.8% (wt/vol) agarose gels and were visualized under UV light after staining with ethidium bromide. The presence of class 3 integrons was determined by dot blot hybridization. Chromosomal DNA was transferred to a Hybond N+ nylon membrane (Amersham Life Science, Arlington Heights, Ill.) and hybridized to a *intI3* probe generated by PCR using genomic DNA from *Klebsiella pneumoniae* FFUL 22K as the template. Labeling and detection were performed with the ECL Random Prime labeling and detection system (Amersham Life Science) following the manufacturer's instructions.

Typing of each class 1 and class 2 integron was performed by restriction fragment length polymorphism (RFLP) analysis. PCR products corresponding to the amplification of the 5′CS-3′CS region of class 1 integrons, and of the *attI2-orfX* region of class 2 integrons, were purified using a QIAquick PCR purification kit (QIAgen, Hilden, Germany) and further digested with AluI or HaeIII, respectively. The fragments obtained were separated in a 2.5% agarose gel and visualized under UV light after staining with ethidium bromide. Integron types were designed by roman numerals. The subindex indicates the class to which each integron belongs. Amplified DNA fragments corresponding to the variable regions of each distinct class 1 and class 2 RFLP type integrons were sequenced.

Statistic analysis. Statistic significance for comparison proportions was calculated by the chi-square test ($P < 0.05$ was considered to be statistically significant).

RESULTS

Epidemiological background of *E. coli* isolates. Distribution of the studied isolates among the four *E. coli* phylogenetic groups is shown in Table 2. Groups B2 and D (associated with extraintestinal pathogenic *E. coli*) were the most represented among strains associated with the hospital setting (66%), and groups A and B1 (more related to animal or human commensal strains) were the most represented among those of healthy volunteers (68%) ($P < 0.001$) (10, 15). C-ESBL *E. coli* isolates mainly corresponded to genogroup D (50%; $n = 26/52$ isolates), whereas most C-nESBL isolates belonged to the genogroup B2 (44%; $n = 19/43$ isolates) and most V-nESBL belonged to the genogroup A (50%, $n = 19/38$). Differences in the ecovar composition of C-ESBL and C-nESBL *E. coli* clinical isolates regarding the occurrence of the B2 and D groups could be due to the bias created by the origin of the isolates. Incidences of phylogenetic groups A and B1 were similar between ESBL and non-ESBL *E. coli* clinical isolates (21% versus 28% and 10% versus 9%, respectively).

Four different ESBL groups were identified among the C-ESBL group: (i) TEM type (31%), (ii) SHV type (17%), (iii) CTX-M-9/14 (42%), and (iv) CTX-M-10 (10%). ESBL-encoding genes were transferred by conjugation to suitable recipients by strains producing TEM (81%), SHV (100%), CTX-M-9/14 (86%), and CTX-M-10 (100%).

***E. coli* isolates and antibiotic resistance.** The percentages of isolates nonsusceptible to sulfonamide were similar among C-ESBL and C-nESBL isolates and higher than that among V-nESBL isolates (75 to 77% versus 45%, respectively). However, differences in the incidence of nonsusceptibility to other antimicrobials were observed for the two groups of clinical isolates studied, C-ESBL and C-nESBL: streptomycin (73% versus 53%), trimethoprim (60% versus 37%), gentamicin (31% versus 7%), kanamycin (35% versus 12%), chloramphenicol (33% versus 21%), ciprofloxacin (48% versus 28%), and nalidixic acid (56% versus 35%). The occurrence of nonsusceptibility among fecal *E. coli* V-nESBL isolates for the antibiotics mentioned above was not significantly lower than that obtained for the C-nESBL isolates, except for

TABLE 1. Primers sequences and PCR conditions used in this study

Primer	Oligonucleotide sequence (5′ to 3′)	GenBank accession no. or reference (gene)	Position of amplicon	PCR conditions	Reference(s)
TEM-F	ATA AAA TTC TTG AAG AC	36 (bla_{TEM-1})	208–228	1 cycle of 12 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	30
TEM-R	TTA CCA ATG CTT AAT CA		1075–1055		
SHV-F	GGG TTA TTC TTA TTT GTC GC	M59181 (bla_{SHV-1})	58–77	1 cycle of 12 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	30
SHV-R	TTA GCG TTG CCA GTG CTC		988–971		
CTX-M-9-F	GTG ACA AAG AGA GTG CAA CGG	AF174129 (bla_{CTXM-9} in In60)	6339–6359	1 cycle of 12 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 62°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	9, 33
CTX-M-9-R	ATG ATT CTC GCC GCT GAA GCC		7195–7175		
CTX-M-10-F	CCG CGC TAC ACT TTG TGG C	AY598759 ($bla_{CTXM-10}$ in pRYC21)	4924–4942	1 cycle of 12 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	9, 25
CTX-M-10-R	TTA CAA ACC GTT GGT GAC G		5885–5867		
ChuA.1	GAC GAA CCA ACG GTC AGG AT			1 cycle of 12 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 59°C, 30 s at 72°C; 1 cycle of 7 min at 72°C	8
ChuA.2	TGC CGC CAG TAC CAA AGA CA				
YjaA.1	TGA AGT GTC AGG AGA CGC TG				8
YjaA.2	ATG GAG AAT GCG TTC CTC AAC				
TspE4C2.1	GAG TAA TGT CGG GGC ATT CA	AF222188	8–28		8
TspE4C2.2	CGC GCC AAC AAA GTA TTA CG		160–141		
IntI1-F	GGT CAA GGA TCT GGA TTT CG	U49101 (*int1*)	786–766	1 cycle of 12 min at 94°C; 30 cycles of 30 s at 94°C, 30s at 62°C, 1 min at 72°C; 1 cycle of 8 min at 72°C	23
IntI1-R	ACA TGC GTG TAA ATC ATC GTC		303–324		
5′CS	GGC ATC CAA GCA GCA AG	M73819 (class 1 integron)	1190–1206	1 cycle of 12 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C; 1 cycle of 10 min at 72°C	21
3′CS	AAG CAG ACT TGA CCT GA		1342–1326		
IntI2-F	CAC GGA TAT GCG ACA AAA AGG T	L10818 (*int2*)	219–240	Same as for *int1*	23
IntI2-R	GTA GCA AAC GAG TGA CGA AAT G		1007–986		
attI2-F	GAC GGC ATG CAC GAT TTG TA	AY183453 (class 2 integron)	1943–1962	1 cycle of 12 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 58°C, 3.5 min at 72°C; 1 cycle of 10 min at 72°C	This work
orfX-R	GAT GCC ATC GCA AGT ACG AG		4848–4928		42
IntI3-F	AGT GGG TGG CGA ATG AGT G	D50438 (*int3*)	178–196	1 cycle of 12 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C; 1 cycle of 8 min at 72°C	12
IntI3-R	TGT TCT TGT ATC GGC AGG TG		777–758		

chloramphenicol (5%), ciprofloxacin (11%), and nalidixic acid (24%).

Prevalence of integrons in C-ESBL versus C-nESBL and V-nESBL isolates. Prevalence of integrons among the *E. coli* groups studied is shown in Table 2. Class 1 integrons were more frequently found among C-ESBL (67%) than among C-nESBL (40%) or V-nESBL (26%) *E. coli* strains ($P < 0.001$). Among C-ESBL isolates, class 1 integrons were mainly associated with isolates harboring $bla_{CTX-M-9/14}$ (95%) or bla_{SHV} (67%) and less with bla_{TEM} (50%) or $bla_{CTX-M-10}$ (0%). Conjugative transfer of an integron with the genetic elements carrying ESBL *bla* genes was observed more frequently for CTX-M-9/14 (95%) than for SHV (33%)-, TEM (23%)-, or CTX-M-10 (0%)-producing *E. coli*. A number of C-ESBL-producing *E. coli* isolates carried more than one class 1 integron (10%, $n = 5/52$).

The occurrence of class 2 integrons was similar among clinical C-ESBL or C-nESBL and commensal V-nESBL (13%, 12%, and 18%, respectively) *E. coli* isolates. Within the C-ESBL group, these genetic elements were more common

TABLE 2. Prevalence of class 1 and class 2 integrons among *E. coli* isolates from patients at the Hospital Universitario Ramón y Cajal and from healthy volunteers

E. coli isolate group (no. of PFGE types)	Class 1		Class 2		Classes 1 + 2	Strains without integrons	Total % of integrons	Phylogenetic group			
	int1$^+$	Gene cassettes as part of class 1 integrons	*int2*$^+$	Gene cassettes as part of class 2 integrons				A	B1	B2	D
C-ESBL (52)	67[a] (35/52)[b]	62 (32/52)	13 (7/52)	13 (7/52)	8 (4/52)	27 (14/52)	73 (38/52)	21 (11/52)	10 (5/52)	19 (10/52)	50 (26/52)
C-nESBL (43)	40 (17/43)	33 (14/43)	12 (5/43)	12 (5/43)	2 (1/43)	51 (22/43)	49 (21/43)	28 (12/43)	9 (4/43)	44 (19/43)	19 (8/43)
V-nESBL (38)	26 (10/38)	21 (8/38)	18 (7/38)	16 (6/38)	8 (3/38)	63 (24/38)	37 (14/38)	50 (19/38)	18 (7/38)	11 (4/38)	21 (8/38)
Total (133)	47 (62/133)	41 (54/133)	14 (19/133)	14 (18/133)	6 (8/133)	45 (60/133)	55 (73/133)	32 (42/133)	12 (16/133)	25 (33/133)	32 (42/133)

[a] Percentage of isolates.
[b] Number of isolates/total.

among isolates containing bla_{TEM} (19%) than $bla_{CTX\text{-}M\text{-}9/14}$ (14%) or bla_{SHV} (11%), whereas they were absent among isolates containing $bla_{CTX\text{-}M\text{-}10}$ (0%). Class 2 integrons were not cotransferred with ESBL *bla* genes in any case. Simultaneous presence of class 1 and class 2 integrons was detected in all *E. coli* groups (2 to 8%). Gene cassettes were not detected for a number of isolates harboring *intI1* (13%, $n = 8/62$) or *intI2* (5%, $n = 1/19$).

Class 1 integrons were found among all four *E. coli* phylogenetic groups at similar rates (40 to 56%). Class 2 integrons were more common among *E. coli* ecovar D (26%) than among strains of the B1 (13%), A (12%), or B2 (3%) groups (Table 3). Class 3 integrons were not detected in any of the isolates studied.

The prevalence of class 1 integrons in C-ESBL isolates dramatically increased along the studied period from 30% during the 1988 to 1995 period to 87% during 1996 to 1998 and reached 70% in 1999 to 2000. Among C-nESBL isolates, these elements increased from 36% (1996 to 1998) to 41% (1999 to 2000). Class 2 integrons also increased over time.

Diversity of *E. coli* integrons. Seven different class 1 integrons were identified (Table 4). Types I_1, II_1, and IV_1 were the most prevalent groups. Type IV_1, which included a *dfrA16* and an *aadA2* gene cassette, was the most frequently found among C-ESBL clones, due to the high prevalence of $bla_{CTX\text{-}M\text{-}9}$, which is located on In60, an unusual integron that contains the first 5′CS-3′CS region corresponding to type IV_1 (33; T. M. Coque, M. C. Varela, A. Oliver, E. Machado, J. C. Galán, F. Baquero, and R. Cantón, abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstract C2-646, 2002). The gene cassettes most commonly found in our collection were those coding for aminoglycoside and/or trimethoprim resistances. Three class 2 integrons were detected, and their sequences were identical to those described for Tn*1826* (Type I_2), Tn*7* (Type II_2), and Tn*1825* (Type III_2) (Table 4 and Fig. 1) (3). Type II_2 was the most frequent and widely distributed. Type I_2 and type III_2 were only found among isolates of the V-nESBL and C-nESBL groups, respectively.

The association between antibiotic resistance phenotypes and class 1 or class 2 integrons is shown in Table 5. Integrons were frequently found in resistant strains, although the presence of gene cassettes coding for a particular antibiotic resistance was only demonstrated for trimethoprim, sulfonamide, streptomycin, spectinomycin, amikacin, gentamicin, or kanamycin. Nevertheless, genes coding for resistance to the above antibiotics were not always linked to class 1 integrons. Strains susceptible to trimethoprim, sulfonamide, streptomycin, gentamicin, kanamycin, chloramphenicol, ciprofloxacin, and nalidixic acid did not carry class 1 or 2 integrons.

DISCUSSION

In this study, comparative information is provided about the evolution of integron content of ESBL and non-ESBL-producing *E. coli* isolates over more than a decade in a single hospital. The previously described wide dissemination of integrons among both clinical and commensal *E. coli* human strains (11, 19, 23) and the increase of the class 1 integrons rate among nosocomial isolates during the last decade (34, 43) was confirmed.

TABLE 3. Distribution of class 1 and class 2 integrons among different *E. coli* phylogenetic groups

Phylogenetic group	Presence of integrons			
	Class 1 (n = 62)	Class 2 (n = 19)	Classes 1 + 2 (n = 8)	No integrons (n = 60)
A (n = 42)	40[a] (17/42)[b]	12 (5/42)	2 (1/42)	50 (21/42)
B1 (n = 16)	56 (9/16)	13 (2/16)	13 (2/16)	44 (7/16)
B2 (n = 33)	42 (14/33)	3 (1/33)	3 (1/33)	58 (19/33)
D (n = 42)	52 (22/42)	26 (11/42)	10 (4/42)	31 (13/42)

[a] Percentage of isolates.
[b] Number of isolates/total.

Class 1 integrons were more frequently found among *E. coli* isolates associated with the clinical setting than among fecal *E. coli* isolates from healthy human volunteers, suggesting a linkage with particular strains and/or transferable genetic elements in the hospital. In fact, class 1 integrons are frequent in our series among ESBL-producing clinical isolates because of the high proportion of strains containing $bla_{CTX\text{-}M\text{-}9}$, a gene linked to In60, an In6-like class 1 integron (28, 33). CTX-M-9 is one of the most common ESBLs found in Spain, and it has been increasingly recovered from *E. coli* since its first description in 1996 (14, 33). Without this bias, the distribution of class 1 integrons could be considered similar among C-ESBL and C-nESBL isolates (47% versus 40%).

Previous studies have shown the association of ESBL genes with plasmids from bacteria responsible for nosocomial outbreaks and associated with class 1 integrons (39). However, we found a relatively low occurrence of class 1 integrons within different plasmids carrying bla_{TEM} or bla_{SHV} genes (23% and 33%, respectively) and the lack of any integron element on $bla_{CTX\text{-}M\text{-}10}$-producing isolates. Conversely, the simultaneous presence of the same class 1 integron types among C-ESBL and C-nESBL isolates recovered from the same hospital wards might indicate a wide dissemination of specific structures in which integrons are located. Indeed, there is a certain specificity of integrons for particular dispersive units (28, 29, 35, 37, 39). If class 1 integrons are more frequently found among hospital clinical isolates, there is a similar occurrence of class 2 integrons among clinical isolates and fecal human isolates (13 versus 18%) from healthy people, suggesting the current absence of privileged selection for strains carrying class 2 integrons in the nosocomial setting.

A great diversity of ARI has been reported in different environments, and changes in the gene cassette content as a result of different genetic events have been described under high antibiotic selective pressure (26, 37, 43). However, our results indicate a low diversity and stability of class 1 integrons, in agreement with other European studies (22). The most common class 1 integrons were types I_1, II_1, and IV_1. Type I_1, related to that of Tn*21*, and type II_1 were also the most common integrons found among isolates from different continents (21–23, 28, 42). Type IV_1 corresponds to the first 5′CS/3′CS part of In60, in which $bla_{CTX\text{-}M\text{-}9}$ is located, and also to the first 5′CS/3′CS part of In36, containing the *qnr* gene widely disseminated in China (33, 41). Regarding class 2 integrons, we found three out of the five known class 2 integrons, which indicates the occurrence of types other than the widely disseminated Tn*7*, such as Tn*1825* or Tn*1826* (3, 42).

The presence of integrons was independently associated with resistance to trimethoprim, sulfonamide, or streptomycin, in agreement with previous studies (20, 22). Resistance to trimethoprim and sulfonamides is usually determined in integrons (11, 32), but in our series we could not detect integrons in a small proportion of trimethoprim-resistant strains or in 25% of sulfonamide-resistant isolates. We cannot discard the presence of unusual class 1 integron structures escaping classical amplification procedures, or of *sul2* or *sul3* genes, not located in class 1 integrons (13). The finding of a similar rate of nonsusceptibility to sulfonamides among both groups of

TABLE 4. Class 1 and class 2 integrons found among *E. coli* isolates from clinical specimens (ESBL and non-ESBL) and from healthy volunteers

RFLP type	Length of variable region (bp)	Gene cassettes and order	Resistance phenotype[a]	No. of isolates	C-ESBL (n = 52)				C-nESBL (n = 43)	V-nESBL (n = 38)	Isolation date
					TEM	SHV	CT-X-M-9/14	CTX-M-10			
Class 1 integrons											
I_1	1,000	*aadA1*	Sm Sp	15	0	4	3	0	6	2	1988–2001
II_1	1,500	*dhfrA1-aadA1*	Tmp Sm Sp	12	1	2	2	0	5	2	1997–2001
III_1	1,800	*dhfrA12-orfF-aadA2*	Tmp Sm Sp	1	1	0	0	0	0	0	1997
IV_1	1,500	*dhfrA16-aadA2*	Tp Sm Sp	13	0	0	13	0	0	0	1996–2000
V_1	950	*aadA2*	Sm Sp	1	0	0	1	0	0	0	1998
VI_1	1,500	*dhfrA17-aadA5*	Tp Sm Spc	5	1	0	0	0	3	1	1997–2001
VII_1	800	*aac(6′)-Ib″*	Km Ak Tb Nt Gm	2	1	0	0	0	0	1	2000–2001
Class 2 integrons											
I_2	1,600	*sat-aadA1-orfX*	Str Sm Sp	2	0	0	0	0	0	2	2001
II_2	1,900	*dhfrA1-sat-aadA1-orfX*	Tp Str Sm Sp	15	3	0	3	0	5	4	1991–2001
III_2	2,300	*orf-sat-aadA1-orfX*	Str Sm Sp	1	0	1	0	0	0	0	2000

[a] Sm, streptomycin; Sp, spectinomycin; Tp, trimethoprim; Km, kanamycin; Ak, amikacin; Tb, tobramycin; Nt, netilmicin; Gm, gentamicin; Str, streptotricin.

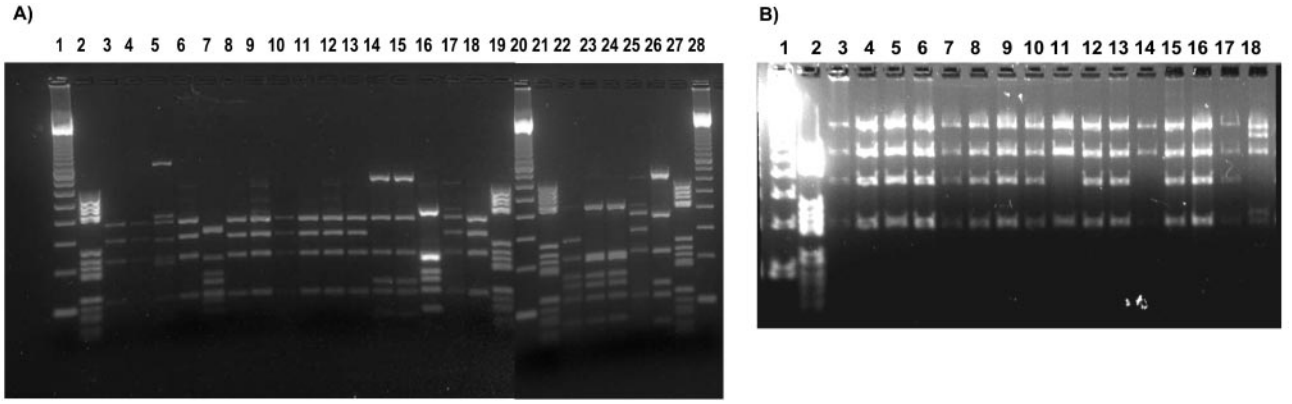

FIG. 1. RFLP analysis of class 1 and class 2 integrons. (A) AluI digest of class 1 integron DNA from *E. coli* isolates: lanes 1, 20, and 28, DNA molecular weight marker λ-EcoT14I/BglII digest (Takara Bio Inc.); lanes 2, 19, 21, and 27, DNA molecular weight marker III (Roche Diagnostics); anes 3, 4, 6, 8 to 13, 17, 18, and 25, type I_1 (*aadA1*); lane 7, type III_1 (*dhfrA12-orfF-aadA2*); lanes 14, 15, and 26, type II_1 (*dhfrA1-aadA1*); lanes 16, 23, and 24, type IV_1 (*dhfrA16-aadA2*); lane 22, type V_1 (*aadA2*). (B) HaeII digest of class 2 integron DNA from *E. coli* isolates: lane 1, DNA molecular weight marker λ-EcoT14I/BglII digest (Takara Bio Inc.); lane 2, DNA molecular weight marker III (Roche Diagnostics); lanes 3 to 10, 12, 13, and 15 to 17, type II_2 (*dhfrA1-sat-aadA1-orfX*); lanes 11 and 14, type I_2 (*sat-aadA1-orfX*); lane 18, type III_2 (*orf-sat-aadA1-orfX*).

clinical *E. coli* isolates was surprising, since the occurrence of class 1 integrons in C-ESBL isolates was much higher than among C-nESBL isolates. Again, that could be explained by a higher frequency of *sul2* and *sul3* genes in strains belonging to ecovar B2 (13), predominant in the C-nESBL group. The *aad* genes were also extremely common in our series and were never located at the first position in the integron platform when other gene cassettes were present, suggesting its earlier recruitment by the element (26). Apart from sulfonamides, trimethoprim, or streptomycin-spectinomycin resistance, we only found gene cassettes coding for kanamycin and gentamicin resistance.

As in other recent studies, resistance to quinolones was more common among integron-containing strains (20). This association could be explained in part by the putative presence of the *qnr* gene that may be located on an In6-like class 1 integron (41). However, the absence of ORF513 in such strains precludes the presence of this gene as part of an In6-like structure (unpublished results). Finally, most chloramphenicol-resistant strains contained a class 1 integron (82%). Whether genes coding for resistance to this antibiotic are part of an unusual class 1 integron or part of individual cassettes integrated in the chromosome as previously described in old studies deserves further research.

Our results indicate that the selection and spread of genetic elements encoding ESBL has not a major role for integron dispersal, except when bla_{ESBL} genes are within an integron platform, such as $bla_{CTX\text{-}M\text{-}9}$. In addition, the contribution of integrons to ESBL dissemination seems to be of small significance, as the encoded resistances currently correspond to old antibiotics without significant intensity of selective pressure in the current clinical setting. This situation might eventually change, following possible events of selection of specific broad-spectrum plasmids able to capture integrons (7, 35), or by integron capture of determinants encoding resistances to antibiotics frequently used in the hospital environment (41). Surveillance of the integron content of nosocomial *E. coli* populations may be critical to predict and prevent the spread of particular antibiotic resistance determinants.

TABLE 5. Association between antibiotic resistance and presence of class 1 or class 2 integrons among *E. coli* isolates from clinical and community environments

Antibiotic[a]	No. of resistant strains	No. of strains with integrons	Class 1		Class 2		No. of strains without integrons
			intI1^{+}[b]	Resistance linked to class 1 integrons[c]	*intI2*$^{+}$[b]	Resistance linked to class 2 integrons[c]	
Tp	59	58	51	29	15	15	1
Su	89	67	61	61	14	0	22
Sm	82	60	50	34	18	18	22
Gm	21	15	13	2	6	0	6
Km	26	21	19	1	5	0	5
Cm	28	23	23	0	2	0	5
Cp	41	33	29	0	9	0	8
Na	53	39	34	0	11	0	14

[a] Tp, trimethoprim; Su, sulfonamides; Sm, streptomycin; Gm, gentamicin; Km, kanamycin; Cm, chloramphenicol; Cp, ciprofloxacin; Na, nalidixic acid.
[b] Number of isolates containing the *intI1* or *intI2* gene.
[c] Number of isolates with gene cassettes encoding resistance to the corresponding antibiotic.

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REFERENCES

1. **Arakawa, Y., M. Murakami, K. Suzuki, H. Ito, R. Wacharotayankun, S. Ohsuka, N. Kato, and M. Ohta.** 1995. A novel integron-like element carrying the metallobetalactamase gene bla_{IMP}. Antimicrob. Agents Chemother. **39:**1612–1615.
2. **Baquero, F.** 2004. From pieces to patterns: evolutionary engineering in bacterial pathogens. Nat. Rev. Microbiol. **2:**510–518.
3. **Birski, L., and D. Mazel.** 2003. Erythromycin esterase gene *ere*(A) is located in a functional gene cassette in an unusual class 2 integron. Antimicrob. Agents Chemother. **47:**3326–3331.
4. **Bonnet, R.** 2004. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. **48:**1–14.
5. **Bradford, P. A.** 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. **14:**933–951.
6. **Cantón, R., T. M. Coque, and F. Baquero.** 2003. Multi-resistant Gram-negative bacilli: from epidemics to endemics. Curr. Opin. Infect. Dis. **16:**315–325.
7. **Carattoli, A., L. Villa, C. Pezzella, E. Bordi, and P. Visca.** 2001. Expanding drug resistance through integron acquisition by IncFI plasmids of Salmonella enterica Typhimurium. Emerg. Infect. Dis. **7:**444–447.
8. **Clermont, O., S. Bonacorsi, and E. Bingen.** 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl. Environ. Microbiol. **66:**4555–4558.
9. **Coque, T. M., A. Oliver, J. C. Perez-Diaz, F. Baquero, and R. Canton.** 2002. Genes encoding TEM-4, SHV-2, and CTX-M-10 extended-spectrum β-lactamases are carried by multiple *Klebsiella pneumoniae* clones in a single hospital (Madrid, 1989 to 2000). Antimicrob. Agents Chemother. **46:**500–510.
10. **Duriez, P., O. Clermont, S. Bonacorsi, E. Bingen, A. Chaventré, J. Elion, B. Picard, and E. Denamur.** 2001. Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. Microbiology **147:**1671–1676.
11. **Fluitz, A. C., and F. J. Schmitz.** 1999. Class 1 integrons, gene cassettes, mobility and epidemiology. Eur. J. Clin. Microbiol. Infect. Dis. **18:**761–770.
12. **Goldstein, C., M. S. Lee, S. Sanchez, et al.** 2001. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents Chemother. **45:**723–726.
13. **Grape, M., L. Sundstrom, and G. Kronvall.** 2003. Sulphonamide resistance gene *sul3* found in *Escherichia coli* isolates from human sources. J. Antimicrob. Chemother. **52:**1022–1024.
14. **Hernández, J. R., L. Martínez-Martínez, R. Cantón, T. M. Coque, A. Pascual, and the Spanish Group for Nosocomial Infections (GEIH).** 2005. Nationwide study of *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β-lactamases in Spain. Antimicrob. Agents Chemother. **49:**●●●●–●●●●.
15. **Herzer, P. J., S. Inouye, M. Inouye, and T. S. Whittam.** 1990. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. J. Bacteriol. **172:**6175–6181.
16. **Hochhut, B., Y. Lofti, D. Mazel, S. M. Faruque, R. Woodgate, and M. K. Waldor.** 2001. Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. Antimicrob. Agents Chemother. **45:**2991–3000.
17. **Jacoby, G. A., and L. Sutton.** 1991. Properties of plasmids responsible for production of extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **35:**164–169.
18. **Kaufmann, M. E.** 1998. Pulsed-field gel electrophoresis, p. 17–31. *In* N. Woodford and A. P. Johnson (ed.), Methods in molecular medicine, vol 15. Molecular bacteriology: protocols and clinical applications. Humana Press Inc., Totowa, N.J.
19. **Leverstein-van Hall, M. A., A. Paauw, A. T. A. Box, H. E. M. Blok, J. Verhoef, and A. C. Fluit.** 2002. Presence of integron-associated resistance in the community is widespread and contributes to multidrug resistance in the hospital. J. Clin. Microbiol. **40:**3038–3040.
20. **Leverstein-van Hall, M. A., H. E. M. Blok, R. T. Donders, A. Paauw, A. C. Fluit, and J. Verhoef.** 2003. Multidrug resistance among *Enterobacteriaceae* is strongly associated with the presence of integrons and is independent of species or isolate origin. J. Infect. Dis. **187:**251–259.
21. **Levesque, C., L. Piche, C. Larose, and P. H. Roy.** 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. **39:**185–195.
22. **Martinez-Freijo, P., A. C. Fluit, F.-J. Schmitz, J. Verhoef, and M. E. Jones.** 1999. Many class I integrons comprise distinct stable structures occurring in different species of *Enterobacteriaceae* isolated from widespread geographic regions in Europe. Antimicrob. Agents Chemother. **43:**686–689.
23. **Mazel, D., B. Dychinco, V. A. Webb, and J. Davies.** 2000. Antibiotic resistance in the ECOR collection: integrons and identification of a novel *aad* gene. Antimicrob. Agents Chemother. **44:**1568–1574.
24. **National Committee for Clinical Laboratory Standards.** 2000. Methods for diffusion disk antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
25. **Oliver, A., T. M. Coque, D. Alonso, A. Valverde, F. Baquero, and R. Cantón.** 2005. CTX-M-10 linked to a phage-related element is widely disseminated among *Enterobacteriaceae* in a Spanish hospital. Antimicrob. Agents Chemother. **49:**1567–1571.
26. **Partridge, S. R., C. Collis, and R. M. Hall.** 2002. Class 1 integrons containing a new cassette, *aadA10*, with Tn*1404* from R151. Antimicrob. Agents Chemother. **46:**2400–2408.
27. **Partridge, S. R., G. D. Recchia, H. W. Stokes, and R. M. Hall.** 2001. Family of class 1 integrons related to In4 from Tn*1696*. Antimicrob. Agents Chemother. **45:**3014–3020.
28. **Partridge, S. R., H. J. Brown, H. W. Stokes, and R. M. Hall.** 2001. Transposons Tn*1696* and Tn*21* and their integrons In4 and In2 have independent origins. Antimicrob. Agents Chemother. **45:**1263–1270.
29. **Preston, K. E., E. M. Graffunder, A. M. Evans, and R. A. Venezia.** 2003. Survey of plasmid-associated genetic markers in *Enterobacteriaceae* with reduced susceptibilities to cephalosporins. Antimicrob. Agents Chemother. **47:**2179–2185.
30. **Rasheed, J. K., C. Jay, B. Metchock, F. Berkowitz, L. Weigel, J. Crellin, C. Steward, B. Hill, A. A. Medeiros, and F. C. Tenover.** 1997. Evolution of extended-spectrum beta-lactam resistance (SHV-8) in a strain of *Escherichia coli* during multiple episodes of bacteremia. Antimicrob. Agents Chemother. **41:**647–653.
31. **Rosser, S. J., and H.-K. Young.** 1999. Identification and characterization of class 1 integrons in bacteria from the aquatic environment. J. Antimicrob. Chemother. **44:**11–18.
32. **Rowe-Magnus, D. A., and D. Mazel.** 2002. The role of integrons in antibiotic resistance gene capture. Int. J. Med. Microbiol. **292:**115–125.
33. **Sabaté, M., R. Tarragó, F. Navarro, E. Miró, C. Vergés, J. Barbé, and G. Prats.** 2000. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-9) from *Escherichia coli* in Spain. Antimicrob. Agents Chemother. **44:**1970–1973.
34. **Schmitz, F. J., D. Hafner, R. Geisel, P. Follmann, K. Kirchke, K. Köhrer, J. Verhoef, and A. C. Fluit.** 2001. Increased prevalence of class 1 integrons in *Escherichia coli*, *Klebsiella* species, and *Enterobacter* species isolates over a 7-year period in a German university hospital. J. Clin. Microbiol. **39:**3724–3726.
35. **Sorum, H., T. M. Lábee-Lund, A. Solberg, and A. Wold.** 2003. Integron-containing IncU plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. Antimicrob. Agents Chemother. **47:**1285–1290.
36. **Sutcliffe, J. G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75:**3737–3741.
37. **Tennstedt, T., R. Szczepanowski, S. Braun, A. Pühler, and A. Sclüter.** 2003. Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. FEMS Microbiol. Ecol. **45:**239–252.
38. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
39. **Villa, L., C. Pezzella, F. Tosini, P. Visca, A. Petrucca, and A. Carattoli.** 2000. Multiple-antibiotic resistance mediated by structurally related IncL/M plasmids carrying an extended-spectrum beta-lactamase gene and a class 1 integron. Antimicrob. Agents Chemother. **44:**2911–2914.
40. **Wang, M., D. F. Sahm, G. A. Jacoby, and D. C. Hooper.** 2004. Emerging plasmid-mediated quinolone resistance associated with the *qnr* gene in *Klebsiella pneumoniae* clinical isolates in the United States. Antimicrob. Agents Chemother. **48:**1295–1299.
41. **Wang, M., J. H. Tran, G. A. Jacoby, Y. Zhang, F. Wang, and D. C. Hooper.** 2003. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob. Agents Chemother. **47:**2242–2248.
42. **White, P. A., C. J. McIver, and W. D. Rawlinson.** 2001. Integrons and gene cassettes in the *Enterobacteriaceae*. Antimicrob. Agents Chemother. **45:**2658–2661.
43. **Yu, H. S., J. C. Lee, H. Y. Kang, D. W. Ro, J. Y. Chung, Y. S. Jeong, S. H. Tae, C. H. Choi, E. Y. Lee, S. Y. Seol, Y. C. Lee, and D. T. Cho.** 2003. Changes in the gene cassettes of class 1 integrons among *Escherichia coli* isolates from urine specimens collected in Korea during the last two decades. J. Clin. Microbiol. **41:**5429–5433.